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COL4A3 mutations and their clinical consequences in thin basement membrane nephropathy (TBMN)[1]

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***COL4A3* mutations and their clinical consequences in thin basement membrane nephropathy (TBMN).**

Background. Thin basement membrane nephropathy (TBMN) is often caused by mutations in the *COL4A3* and *COL4A4* genes.

Methods. We examined 62 unrelated individuals diagnosed with TBMN by renal biopsy ($N = 49$, 79%) or a positive family history of hematuria but without a biopsy ($N = 13$, 21%) for mutations in the *COL4A3* gene and the *COL4A3/COL4A4* promoter. All 52 exons of *COL4A3* as well as the *COL4A3/COL4A4* promoter were screened with single-stranded conformational polymorphism (SSCP) analysis at 4°C and at room temperature. Amplicons that demonstrated electrophoretic abnormalities were sequenced.

Results. Seven mutations were demonstrated in seven patients: G532C and G584C in exon 25, G596R in exon 26, G695R in exon 28, and IVS 2224 − 11C>T, IVS 2980 + 1G>A and IVS 3518 – 7C>G. No mutations were found in the *COL4A3/COL4A4* promoter. Four novel polymorphisms or variants (P116T in exon 6, P690P in exon 27, and G895G and A899A in exon 33) were also demonstrated. In addition, P1109S and Q1495R, which had been described previously but whose status was unclear, were shown to be polymorphisms. All seven mutations described here were associated with hematuria. While one mutation (2980 + 1G>A) was found in an individual who also had proteinuria, none of her family members with the same mutation had increased urinary protein. None of the patients with these seven mutations had renal impairment. Hematuria was completely penetrant in families with the G532C, G584C, G596R, and IVS 2980 + 1G>A mutations but not with the G695R and IVS 3518 − 7C>G mutations.

Conclusion. COL4A3 mutations are common in TBMN.

Thin basement membrane nephropathy (TBMN) occurs in at least 1% of otherwise normal children and adults [1, 2], and is usually characterized by persistent dysmorphic hematuria, minimal proteinuria, normal renal function, and an excellent prognosis. Ultrastructural examination of the renal biopsy in TBMN demonstrates a uniformly thinned glomerular basement membrane (GBM) [3]. Two thirds of affected individuals have another family member with hematuria [4] and inheritance in these families is autosomal dominant [5].

The demonstration of a thinned GBM in heterozygotes from families with autosomal-recessive Alport syndrome first suggested that TBMN might represent the carrier state for this condition or at least be caused by mutations in the same genes [abstract; Lambrecht R, et al, *J Am Soc Nephrol* 7:1616A, 1996] [6–8]. Our laboratory subsequently showed that nearly 40% of families with biopsy-proven TBMN have hematuria that segregates with the *COL4A3/COL4A4* locus [4]. Furthermore, we demonstrated identical *COL4A4* mutations in carriers of autosomal-recessive Alport syndrome and in TBMN [9, 10] which confirmed that TBMN sometimes represents the carrier state for autosomal-recessive Alport syndrome.

A number of different *COL4A3* and *COL4A4* mutations have now been described in TBMN [9, 11–14]. The aim of the present study was to demonstrate the *COL4A3* mutations that caused TBMN in a cohort of patients who had already been examined for *COL4A4* mutations, to determine whether mutations occurred in the *COL4A3/COL4A4* promoter, and to correlate these mutations with clinical features.

METHODS

Patients

Sixty-two unrelated patients with TBMN were studied. All had ≥ 30,000 dysmorphic red blood cells/mL on phase-contrast microscopy of a midstream urine specimen [15] on at least two occasions. None had a clinically detectable hearing loss, and none knew of another family member with X-linked or autosomal-recessive Alport syndrome, renal failure, or inherited hearing loss.

In 49 patients (79%), the diagnosis of TBMN was confirmed with the ultrastructural demonstration of diffuse GBM thinning on renal biopsy, and an average GBM width <250 nm calculated from 50 random measurements. None of the biopsies had the lamellation

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typical of X-linked Alport syndrome. In 13 cases (21%), the diagnosis of TBMN was confirmed on a positive family history of hematuria alone.

The presence of hypertension, the level of proteinuria, and the serum creatinine were noted in the index cases at presentation and at follow-up, and the presence of hematuria and proteinuria were noted in the family members participating in the study.

Linkage to *COL4A3/COL4A4* and *COL4A5* loci

The families of 42 patients (68%) were examined for linkage of hematuria to the *COL4A3/COL4A4* locus using methods described previously [4]. Some of these families have been reported before [4]. Briefly, affected family members were identified because they had ≥30,000 dysmorphic red blood cellls/mL on phase-contrast urinary microscopy. DNA from affected and unaffected family members was extracted from peripheral blood leukocytes or buccal brushings, and amplified by polymerase chain reaction (PCR) to determine linkage to the *COL4A3/COL4A4* locus using the CA11, D2S351, D2S401, and PAX3 microsatellite markers and the *COL4A4* Hae III intragenic restriction fragment length polymorphism [16, 17]. Haplotypes were then constructed at the *COL4A3/COL4A4* locus for family members. Families were usually too small for formal linkage studies and logarithm of odds (LOD) scores to be meaningful, but the probabilities of hematuria segregating with the putative disease haplotypes by chance alone were calculated. Linkage to the *COL4A5* locus was excluded using the 2B20, 2B6, and DXS456 microsatellite markers [18, 19].

Screening for mutations in the *COL4A3* exons

For each patient, all 52 *COL4A3* exons were amplified using primers described previously [20] and screened for mutations using single-stranded conformational polymorphism (SSCP) analysis at both room temperature and 4°C [21]. Patients were examined for mutations regardless of whether or not their families were examined and hematuria segregated with the *COL4A3/COL4A4* locus.

Screening for mutations in the *COL4A3/COL4A4* promoter

The region examined began 12 nucleotides upstream of the *COL4A3* start codon and extended 29 bp into intron 1 of the *COL4A4* gene [22, 23]. It was amplified in three parts using the following sets of primers: 5′-CTC AGA GCC TGG GCG AGT CC-3′ and 5′-GAC ACT GCC TGG TAA GTT GG-3′; 5′-GGA AGA CGT GGC TCA GGT CC-3′ and 5′-TCC CGT TAA TCT GGG CAG G-3′; and 5′-GGC CCT GCC CAG ATT AAC GG-3′ and 5′-AGC GGT TGC CCC ACC TAT GG-3′. The annealing temperatures for these reactions were 60°C, 61°C, and 58°C and the products were 244 bp, 307 bp, and 268 bp, respectively.

SSCP analysis

Both forward and reverse primers for each exon were end-labelled with γ^{32}P-adenosine triphosphate (ATP) (Geneworks, Adelaide, Australia) using T4 polynucleotide kinase (Geneworks). PCR amplification was performed in a 5μL reaction containing 25 ng of each labelled primer, 1 mmol/L deoxynucleotide triphosphate (dNTP) (Pharmacia, Uppsala, Sweden), 0.5 μL 10 × PCR × amplification buffer (Gibco BRL, Life Technologies, Gaithersburg, MD, USA) 0.5 μL 10 × PCR × enhancer solution (Invitrogen, Carlsbad, CA, USA), 1.25 mmol/L MgCl2, 0.5 U Taq polymerase (Geneworks), and 20 ng of genomic DNA from index patients. After the reaction was complete, 25 μL of SSCP loading buffer (95% formamide, 5 mmol/L NaOH, 0.1% bromophenol blue, and 0.1% xylene cyanol) was added to each tube, and the samples heated to 95°C for 5 minutes, and chilled on ice. Five microliters from each tube was then loaded onto a 6% nondenaturing polyacrylamide gel, and electrophoresed in 0.6 × TBE at 2 to 10 W for 3 to 16 hours (depending on the size of the fragment) at 4°C. Gels were exposed to x-ray film for 1 to 20 hours at −80°C.

Sequencing

All amplicons that demonstrated band shifts were reamplified (Big Dye Terminator; Perkin-Elmer, Warrington, UK), purified, and sequenced in both directions using an automated DNA sequencer. Where a variant was found, DNA samples from all available family members were sequenced to determine whether the variant segregated with hematuria. Mutations were considered pathogenic if they changed the amino acid sequence but were not present in 50 nonhematuric normals.

This project was approved by the Human Research Ethics Committee of the Austin and Repatriation Medical Centre, and all participants provided signed, informed consent to the study.

RESULTS

Mutations and polymorphisms in the *COL4A3* gene

Seven novel *COL4A3* mutations were demonstrated in seven of the 62 patients with TBMN (11%) (Table 1). These were G532C and G584C in exon 25, G596R in exon 26, G695R in exon 28, and IVS 2224 − 11C>T, IVS 2980 + 1G>A and IVS 3518 − 7C>G. None of these changes was present in 50 nonhematuric normals.

We also demonstrated four novel polymorphisms or variants (Table 2). These were P116T in exon 6, P690P in exon 27, and G895G and A899A in exon 33. P1109S

Table 1. Novel mutations in the *COL4A3* gene in thin basement membrane nephropathy (TBMN)

Exon	Mutation	Effect on coding sequence	Clinical features in index case	Hematuria segregates with mutation in family	Frequency in controls
25	1594G>T	G532C	F 24; 240,000 RBC/mL; proteinuria <200 mg/L; followed for 12 years	Yes	0/50
	1750G>T	G584C	F 54; 135,000 RBC/mL; proteinuria < 200 mg/L, followed for 6 years	Yes	0/50
26	1786G>C	G596R	F 49; 74,000 RBC/mL; proteinuria <200 mg/L; followed for 19 years	Yes	0/50
28	2083G>A	G695R	F 30; 100,000 RBC/mL; proteinuria < 200 mg/L; followed for 13 years	No	0/50
30	2224 – 11C>T	IVS	F 50; 50,000 RBC/mL; proteinuria <200 mg/L; not followed	Not studied	0/50
35	2980 + 1G>A	IVS	F 16; 160,000 RBC/mL; proteinuria 2.7 g/day; followed for 2 years	Yes	0/50
41	3518 – 7C>G	IVS	F 42; 50,000 RBC/mL; proteinuria < 200 mg/L; followed for 5 years	No	0/50

Abbreviations are: RBC, red blood cells; IVS, intervening sequence.

Table 2. Polymorphisms in the *COL4A3* gene

Exon	Polymorphism	Effect on coding sequence	Heterozygosity index of novel polymorphisms
2	IVS2 + 12C>A		
	127G>C	G43R	
6	**346C>A**	**P116T**	**0.02**
7	**422C>T**	**P141L**	
9	473C>A	A158D	
	485G>A	G162E	
15	878C>G	P293R	
17	976G>T	D326Y	
21	1195T>C	L399L	
	1223G>A	R408H	
22	1352A>G	H451R	
23	1452G>A	G484G	
25	1721T>C	L574P	
27	**2071T>A**	**P690P**	**0.03**
33	**2685A>C**	**G895G**	**0.3**
	2697C>A	**A899A**	**0.3**
38	**3325C>T**	**P1109S**	**0.01**
40	IVS39 + 18delA		
43	3807C>A	D1269E	
48	4421T>C	L1474P	
49	**4484A>G**	**Q1495R**	**0.05**

Novel polymorphisms are in bold.

had been described previously as a rare variant of uncertain significance but we found it in two patients with TBMN and a nonhematuric normal. We also demonstrated Q1495R, which was previously considered to be a mutation, in one patient and in one of the 10 normals whose DNA was sequenced. We therefore consider both P1109S and Q1495R are polymorphisms. (This is despite some patients with TBMN having no hematuria [24] and none of the nonhematuric "normals" having had a renal biopsy to exclude TBMN). The heterozgosity indices for the novel polymorphisms are also shown in Table 2.

No mutations or polymorphisms were demonstrated in the *COL4A3/COL4A4* promoter region.

Clinical features associated with mutations

All seven index cases in whom mutations were identified had hematuria (median 135×10^3 red blood cells/mL, range 50 to 660) at presentation (Table 1). Only one had proteinuria >500 mg/day, and this was a 16-year-old girl with IVS 2980 + 1G>A and 2.7 g protein/day. None of her three relatives who also had the IVS 2980 + 1G>A mutation had proteinuria >500 mg/day. This girl has subsequently had a renal biopsy, which showed focal glomerulosclerosis and glomerular membrane irregularity and lamellation on electron microscopy that suggested Alport syndrome. Hematuria was present only in the index case's father and his family and not in any of her maternal relatives. X-linked Alport syndrome had been excluded by linkage studies but this girl may still have had autosomal-dominant or recessive (assuming her mother's disease was subclinical) Alport syndrome.

None of the index cases had hypertension or renal impairment at presentation or increased proteinuria, hypertension, or renal impairment at review after a median of 5 years (range 2 to 12 years).

The families of the index cases with six of the seven mutations were studied. Hematuria was present in all family members with the G532C, G584C, G596R, and IVS 2980 + 1G>A mutations but was present in only one of two and one of four family members with the G695R or IVS 3518 – 7 C>G mutations, respectively.

One patient was of particular interest because she had a definite mutation in the *COL4A3* gene (G695R in exon

28) as well as a possible mutation in the *COL4A4* gene (G999E in exon 33). Glycine substitutions are usually pathogenic but the status of G999E is not clear. The patient was a 30-year-old female with 100,000 urinary red blood cells/mL, 330 mg/L proteinuria, a blood pressure of 130/70 mm Hg, and serum creatinine of 0.06 mmol/L (normal <0.12 mmol/L). She had clinically normal hearing and no lenticonus or retinopathy. Her renal biopsy contained 12 glomeruli, three of which were completely sclerosed, and her GBM was uniformly thinned.

DISCUSSION

We have demonstrated seven novel *COL4A3* mutations in seven patients with TBMN, as well as four novel polymorphisms or variants. In addition, we have shown that P1109S and Q1495R, which were described previously as a variant of uncertain significance and a mutation, respectively, were actually polymorphisms.

All 62 patients studied had clinical features suggesting TBMN and nearly 80% had this diagnosis confirmed on renal biopsy. None of the patients had a family history of autosomal-recessive Alport syndrome, and patients were examined for *COL4A3* mutations regardless of whether or not their families were studied or hematuria within their families segregated with the *COL4A3/COL4A4* locus. X-linked Alport syndrome was excluded on clinical features, family history, GBM ultrastructural appearance, and linkage studies.

Although the pathogenicity of any *COL4* sequence variant is difficult to confirm without functional studies, it is usually considered sufficient to show the variant does not occur in 50 nonhematuric normals. None of the seven mutations described here occurred in normals on SSCP. In addition, all four mutations found in the *COL4A3* coding sequence resulted in glycine substitutions within the collagenous domain. Mutations in the *COL4* genes affecting glycine are usually pathogenic because the substitution of glycine with larger, more highly charged residues disrupts the collagen heterotrimer formation. The other three mutations affected intervening sequences. While it is difficult to be sure of their pathogenicity, 25% mutations in X-linked Alport syndrome affect splice sites in the intervening sequences of the *COL4A5* gene [25], and splice site mutations are probably also common in the the *COL4A3* and *COL4A4* genes.

None of the *COL4A3* mutations we found had been described previously in the homozygous or compound heterozygous forms in autosomal-recessive Alport syndrome. As well as causing TBMN, heterozygous *COL4A3* and *COL4A4* mutations cause autosomal-dominant Alport syndrome [16, 26, 27]. Autosomal-dominant Alport syndrome is characterised by renal failure, hearing loss, and a lamellated GBM, which contrasts with the hematuria, normal renal function, and thinned GBM found in TBMN. Too few mutations have been described in autosomal-dominant Alport syndrome and TBMN to identify the features that distinguish between these conditions.

Polymorphisms are common in the *COL4A3* and *COL4A4* genes, and more than 20 *COL4A3* polymorphisms have been described to date [12, 13, 20]. It is important to identify all these because they may be mistaken for mutations and patients then misdiagnosed with TBMN when they actually have X-linked Alport syndrome.

In this study, no mutations or polymorphisms were identified in the region of the *COL4A3/COL4A4* promoter. Mutations in the promoter and other regulatory sequences of the *COL4A5* gene are also uncommon in X-linked Alport syndrome. Three reports examining the *COL4A5* promoter region have yielded only one mutation so far, and this resulted in a C to T substitution 113 bp upstream of the 5′ end of exon 1′ of *COL*4A6 [abstract; Kawai S, et al, *J Am Soc Nephrol* 7:1615A, 1996] [28, 29]. The *COL4A3/COL4A4* promoter has neither been studied previously in TBMN, nor in the autosomal-recessive and dominant forms of Alport syndrome.

The detection rate of *COL4A3* mutations in the 62 patients with TBMN studied here was low (11%, 7/62) compared with the 50% expected if half the mutations affected the *COL4A3* gene and half the *COL4A4* gene. The rate could be attributed in part to the use of SSCP, which detects only 80% mutations under optimal conditions, and which does not demonstrate larger deletions and splice site mutations when the primers are near exon-intron boundaries. The rate might also be due to the use of primers that were not designed specifically to detect splice site mutations. Splice site mutations are common in X-linked Alport syndrome, but primer design has proven to be a major limitation to their demonstration [25, 28, 29].

However the mutation detection rate increased to 35% (7 *COL4A3* and 9 *COL4A4* mutations in 46 patients) when both the *COL4A3* and *COL4A4* genes were examined. The rate increased further still to 67% (three *COL4A3* and three *COL4A4* mutations in nine families) when only individuals from families where TBMN segregated with the *COL4A3/COL4A4* locus were examined for mutations in these genes. Some individuals from families where hematuria does not segregate with the *COL4A3/COL4A4* locus will still have mutations in these genes because the hematuria is incompletely penetrant [14]. Nevertheless our observation that few *COL4A3* or *COL4A4* mutations are demonstrated when hematuria does not segregate with the *COL4A3/COL4A4* locus suggests the possibility of another genetic locus for TBMN.

All seven index cases with TBMN in whom mutations were identified had hematuria but only one had marked

proteinuria. None of the index cases with these seven mutations had hypertension or renal impairment at presentation or at review. The absence of proteinuria in the family members of the patient with the 2980 + 1G>A mutation implies that other genetic and environmental factors contributed to its demonstration in the index case. Likewise, two mutations (G695R, IVS 3518 – 7 C>G) were not necessarily associated with hematuria in all family members. Finally, we described an individual with possible compound heterozygous mutations in the *COL4A3* and *COL4A4* genes whose clinical features were consistent with TBMN rather than with autosomal-recessive Alport syndrome.

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